



Rotation of treatments between spinosad and amitraz for the control of *Rhipicephalus (Boophilus) microplus* populations with amitraz resistance

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We dedicate this work to David Kemp, who made an invaluable contribution to this project. Largely responsible for the design of the study, he was also one of the most consistent participants in the field work. Without him the project would not have happened, and we have lost a great colleague with his passing. It should be noted that he would have preferred it if we had referred to the subject of this study as *Boophilus microplus*.

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Amitraz

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ABSTRACT

A farmlet study was conducted over 4 years in which three treatments were applied to six groups of Holstein dairy calves. Calves in each group were infested with equal numbers of N-strain (susceptible) and Ultimo strain (amitraz and synthetic pyrethroid resistant) tick larvae to establish self-sustaining populations with an initial, measurable level of resistance to amitraz. Standard counts of all ticks between 4.5 and 8.0 mm diameter on one side of each animal were made each week and treatment was applied when tick numbers exceeded a threshold of 25 engorged adults per side. The three treatments were: 1, spinosad spray whenever tick numbers exceeded the threshold; 2, amitraz spray whenever tick numbers exceeded the threshold; 3, spinosad whenever tick numbers exceeded the threshold for the first 2 months, then amitraz for 2 months, with alternation every subsequent 2 months. Engorged adult female ticks were collected from each treatment group on 10 or 11 occasions during the study and tested using the larval packet test bioassay (LPT) for acaricide resistance. Spinosad 250 ppm provided effective control of amitraz-resistant tick populations in the field, using a similar number of treatments as in the amitraz and rotation groups. The initial infestations of all of the groups resulted in the establishment of populations with *in vitro* evidence of resistance to amitraz using the LPT. Treatment with spinosad or with a rotation between spinosad and amitraz every 2 months resulted in reduced levels of resistance to amitraz according to the LPT. The animals treated with amitraz alone showed increasing resistance to amitraz according to the LPT each summer and autumn with a return to full or almost full susceptibility to amitraz in early spring in all years. This pattern suggests a relative lack of fitness of amitraz-resistant ticks that might be exploited by using an acaricide rotation strategy.

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1. Introduction

Amitraz is a formamidine acaricide that is applied to cattle in plunge dips and spray races to control the cattle tick *Rhipicephalus (Boophilus) microplus*. It has been in use since 1975 in Australia, and the first case of resistance to the chemical in Australia was reported in 1981 (Nolan, 1981). In Mexico amitraz was introduced in 1986 and the first case of resistance was diagnosed in 2001 (Soberanes

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et al., 2002). Resistance to amitraz is now a problem for cattle producers around the world, being reported as such in Australia (Jonsson and Hope, 2007), Mexico (Rodriguez-Vivas et al., 2006), Brazil (Li et al., 2005), and New Caledonia (Chevillon et al., 2007). The prevalence of resistance to amitraz was recently estimated to be 11% in Australia (Jonsson and Hope, 2007) and 19.4% in Mexico (Rodriguez-Vivas et al., 2006). Until recently there was very little resistance to the product and it was the mainstay of tick control in many countries because of its high efficacy and relative low cost (Jonsson and Matschoss, 1998).

Spinosad is a member of the spinosyn family derived from *Saccharopolyspora spinosa*. It has a different mode of action to amitraz and activates nicotinic acetylcholine receptors and secondarily antagonises gamma-aminobutyric acid receptors (Millar and Denholm, 2007). It is very effective against lepidopteran species and it has been shown to be effective against *R. microplus* (particularly nymphs and larvae) when used at high concentrations (Davey et al., 2001). It is registered for tick control in some countries in Latin America, including Brazil. There are no published reports of resistance to spinosad in *R. microplus* ticks to date.

The relatively slow development and spread of resistance to amitraz in Australia has provided some hope that resistance might be associated with a cost to fitness (Jonsson and Hope, 2007), which might facilitate management of resistance with rotation strategies. Prabhaker et al. (1998) demonstrated that rotation between bifenthrin, endosulfan and chlorpyrifos delayed the development of resistance by 10 generations in *Bemisia argentifolii* (silverleaf whitefly) when compared with continuous treatment strategies. Rotation strategies have been used for the control of horn fly (*Haematobia irritans*) with some success (Byford et al., 1999) and also without much success (Barros et al., 1999). It has been noted that beef and dairy farmers in Australia with confirmed amitraz resistance were able to revert to the effective use of amitraz after a period of one or more seasons using an alternative product (Foil et al., 2004). An anecdotal report of apparent efficacy of a rotation with amitraz to control mixed species of ticks has been published (Kamidi and Kamidi, 2005). However, that report describes a single small farm, without controls, with mixed, unidentified and uncounted tick infestations and without any laboratory confirmation of resistance to amitraz. Thullner et al. (2007) showed that rotation between permethrin and coumaphos delayed the development of resistance in a tick population that initially had a low level of resistance to permethrin *in vitro*. However, until now there have been no reports of controlled field based studies of management of resistance using rotation strategies.

The primary aim of this study was to determine whether the use of a treatment strategy that alternated applications of spinosad and amitraz each generation would enable effective control of ticks when there is already measurable resistance to amitraz. The secondary aim was to determine whether the proportion of amitraz-resistant ticks in the populations declined in the group that



Fig. 1. Satellite photograph (Google Earth) of the trial site showing the paddocks numbered according to group (1,2: spinosad; 3,4: amitraz; 5,6: rotation).

was treated only with spinosad (i.e., in which selection pressure was removed).

2. Materials and methods

2.1. Trial site

The trial was conducted in specially designed and fenced paddocks at the University of Queensland Pinjarra Hills Campus. Fig. 1 is a satellite photograph (Google Earth) of the study site with the paddock (farmlet) boundaries superimposed. Coordinates of the study site are 152°55'15"E, 27°31'39"S, and the elevation is approximately 30 m. Pastures were mixed tropical grasses, predominantly kikuyu, green panic, Johnson grass, Rhodes grass and paspalum. Six approximately equally sized paddocks (~1 ha each) were established to minimise the risk of movements of ticks among paddocks. This was achieved by ensuring that the boundaries between the paddocks were perpendicular to the contours of the land to ensure that there was little or no run-off from one paddock to another in the case of rain; boundaries between paddocks were double-fenced with sufficient space to drive a tractor and slasher between the two fence lines; the ground between and immediately adjacent to the fences was regularly sprayed with glyphosate non-selective herbicide to ensure that there was little or no pasture or weed growth between the paddocks; laneways and yards were set up exclusively for the use of the trial animals and cattle were only moved when essential. Each paddock had a single water trough supplied with water from the town supply, a feed trough for pellets and a hay rack.

For 6 months prior to this study the paddocks were left vacant to minimise tick infestations. Two months before the commencement of the trial the paddocks were intensively grazed by 20 *Bos taurus* cattle with the aim of picking up as many surviving larvae as possible. The resulting infestations were very light and acaricide

resistance assays indicated 5% resistance to synthetic pyrethroids and no evidence of amitraz resistance.

2.2. Animals

Thirty-six month-old Holstein-Friesian (*B. taurus*) dairy calves ranging from 120 to 250 kg were initially recruited for this study. All calves were declared by vendors not to have had previous treatments with endectocides or acaricides, but had all previously been exposed to ticks. On introduction to the trial site, calves were identified with uniquely numbered plastic ear tags. Cattle were weighed and then ranked by weight to enable assignment to treatment group. After the initial infestations, side counts of tick numbers were undertaken on all calves and four calves were exchanged to ensure that the mean tick counts of all groups were similar. As the cattle grew, the number held in each paddock was reduced to four.

Cattle were inspected daily by farm staff for their general well-being and to make sure that no animals had escaped from their paddocks into laneways or other paddocks. For most of the study, cattle required supplementary feeding with hay (Pangola, forage sorghum, and lucerne) and for 1 year they were fed pellets containing monensin to aid in the control of coccidiosis. Hay and pellets were fed out daily or every second day, depending on the availability of pasture. Round-bale feeders were used for the hay and pellets were fed in troughs.

2.3. Ticks

Ticks were obtained from the Queensland Department of Primary Industries and Fisheries (QDPI&F) Yeerongpilly Veterinary Laboratories (YVL). Strains used were the NRFS (non-resistant field strain), which is susceptible to all acaricides, and the Ultimo strain, which is resistant to amitraz and synthetic pyrethroids. Larvae were provided in clear plastic vials, each containing 2500 larvae. Each vial was opened carefully and clipped into a pouch in a purpose-made infesting collar placed around the neck of each calf. One vial of each strain was used for each of the infestations. Infestations were carried out weekly for 3 weeks. Viability of the larvae was checked before application to cattle by breathing on the vial and assessing the subsequent increase in motility of the larvae. Only those vials in which there was a clear and vigorous response to this test were used.

2.4. Acaricide treatments

Acaricides were freshly prepared on each day using calibrated vessels at all times. Extinosad (Elanco Animal Health, 25 g/L spinosad SC) was diluted 1:100 in clean water to give 250 ppm. The product was thoroughly mixed by motorized recirculation for 5 min before use. Taktic EC (Intervet 125 g/L amitraz) was diluted 2:1000 in clean water to give 250 ppm amitraz. Acaricides were thoroughly applied to the entire body surface of cattle held in a race at the cattle yards using a motorized spray and hand held spray wand. The volume used was 8–10 L of made up solution per animal. When changing from spinosad to

amitraz in the spray, the reservoir was flushed with three times the volume of water as of acaricide solution that was used. The last one third of the water was used to wash down the walls of the races where the next group of calves was to be treated. This procedure was also used following the final treatment on any day. All personnel wore appropriate protective clothing and followed the guidelines for application of hazardous substances.

2.5. Tick counts

The standard protocol for a side count, as described by Wharton et al. (1970) was followed. Cattle were restrained in a crush for counting ticks on one side of the body. All standard ticks between 4.5 and 8.0 mm were counted and recorded immediately.

2.6. Acaricide resistance testing

We aimed to test acaricide resistance in each of the first three clearly definable generations of ticks and then from each group at least once each season. Over time the generations of ticks became asynchronous among groups, so there was a spread of up to a month in collection dates corresponding with a given season among groups. Because the threshold for treatment was 25 standard ticks, ticks for the bioassay were collected from animals on the day following the identification of the high burdens and before treatment with the prescribed acaricide. Animals with infestations likely to yield sufficient engorged ticks for resistance testing from the groups to be treated were removed from the paddocks and transported a short distance (approximately 1 km) by truck to be housed in moated pens with slatted floors overnight and collecting baskets. This enabled the collection of sufficient numbers of engorged female ticks for the bioassay. The ticks were collected from the floor of the moated pens and the baskets, washed with tap water, dried, and submitted to the YVL for acaricide resistance testing by the larval packet test (LPT) of Stone and Haydock (1962). Amitraz bioassays were conducted using four concentrations: 0.05, 0.1, 0.5 and 1%, and for synthetic pyrethroids, 0.3% cypermethrin was used.

2.7. Treatments

The three treatments were: 1, spinosad spray whenever side counts exceeded a threshold (the threshold was initially set at 100 ticks on one side in first month in order to ensure the establishment of viable populations in the paddocks, then was set at 25 thereafter); 2, amitraz spray whenever indicated by tick numbers exceeding the thresholds; 3, spinosad whenever indicated by tick numbers exceeding the thresholds for the first 2 months, then amitraz for 2 months, with alternation every 2 months, considered to approximate a single generation. The intergenerational period of the cattle tick in south-eastern Australia ranges from 2 to 3 months (Sutherst and Comins, 1979) with a build up of tick numbers in spring into summer and low numbers over-wintering on the pasture. If any one calf in a treatment group had more

standard ticks per side than the threshold, then all calves in the group were treated with the specified acaricide. Two paddocks were randomly assigned to each of the three treatments, except that two of the same treatments were not permitted to occupy adjacent paddocks.

2.8. Data analysis

The number of acaricide treatments applied to each group was tabulated and means and standard errors of the means were calculated. A one-way analysis of variance was conducted, using treatment (spinosad, amitraz, and rotation) as the factor. Statistical significance was set at $P < 0.05$. The effect of treatment on resistance was evaluated independently at each of 10 time points using one-way analysis of variance with treatment as the factor. The effect of season was determined for each group using one-way analysis of variance, using Tukey t -test for pairwise comparisons.

3. Results

3.1. Tick counts

Populations of ticks were established in all of the paddocks. Tick counts are not informative in this study design because the application of a treatment threshold prevents greatly divergent populations of ticks. For this reason they are not analysed or presented here. The number of acaricide applications needed to meet the threshold requirements is a more effective indicator of the size of the tick populations in each paddock.

3.2. Acaricide applications

Table 1 summarises the number of each type of acaricide applied in each season. The mean total number of applications of acaricide for each group that was required to maintain the number of ticks below the threshold level of 25 standard ticks on one side were similar: spinosad 25.5; amitraz 20; rotation 19 ($P = 0.208$) (Tables 2 and 3). There was no significant association

between season and the number of applications (data not shown).

3.3. Acaricide resistance

Fully engorged female ticks were collected from each group on 10 (one group) or 11 (5 groups) occasions during the trial. The collection G1 (first generation) was made at the time of the first peak of engorged adult female ticks after the original application of larvae and before the application of any acaricides. It indicated the resistance status of the first established population of ticks. G2 was the second generation of ticks identified in the study. Subsequently it became more difficult to clearly identify the generation and collections were once per season.

Figs. 2–4 show the relationship in mean survival in the LPT for amitraz using four concentrations and the cumulative number of field treatments with amitraz for the period prior to each collection. Fig. 5 shows the mean mortality over time using a single, discriminating concentration of cypermethrin (0.3%), over 10 time points.

There are clear differences in the percentage survival over time in the amitraz-treated group compared with the rotation and the spinosad-treated groups. All groups showed high levels of survival when exposed to amitraz at all concentrations except 1% amitraz on the first and second generation collections and there were no significant differences among any of the treatments nor any trends to divergent responses. By *Winter 2004*, the percentage survival of amitraz in the amitraz-treated group began to increase relative to the spinosad and rotation treatments, which were returning to fully susceptible levels, at 0.05 and 0.1% amitraz ($P < 0.1$). In the *Spring 2004* collection, the percentage survival of 0.5 and 1.0% amitraz in the LPT was significantly higher ($P < 0.05$) in the amitraz-treated group. By *Autumn 2005* percentage survival was significantly ($P < 0.005$) higher in the amitraz-treated group at all concentrations. In the *Winter 2005* collection the differences were significant at 0.05% ($P < 0.001$), 0.1 and 1% ($P < 0.05$) and tended to significance at 0.5% amitraz concentration in the ($P < 0.1$). *Spring 2005* saw a return of the amitraz-treated group to

Table 1

Number of treatments applied to each group in response to infestations heavier than 25 standard ticks on one side (S = spinosad; A = amitraz) in each season. Because of the small number of treatments in each season for each group, no attempt has been made to compare treatment number within year or season.

Season	Group 1 Spinosad	Group 2 Spinosad	Group 3 Amitraz	Group 4 Amitraz	Group 5		Group 6	
					S	A	S	A
Summer 03–04	1	1	0	1	1	0	0	0
Autumn 04	3	4	2	2	1	2	2	1
Winter 04	1	4	2	3	2	0	2	1
Spring 04	1	2	1	1	0	1	0	1
Summer 04–05	4	4	3	3	1	1	1	0
Autumn 05	3	6	3	3	1	1	1	1
Winter 05	2	0	1	1	1	0	2	0
Spring 05	1	1	2	2	1	1	1	1
Summer 05–06	2	5	3	2	2	1	0	2
Autumn 06	3	2	2	2	2	0	2	0
Winter 06	1	0	1	0	0	1	0	1
Total treatments	22 Spinosad	29 Spinosad	20 Amitraz	20 Amitraz	12 Spinosad, 8 amitraz		10 Spinosad, 8 amitraz	

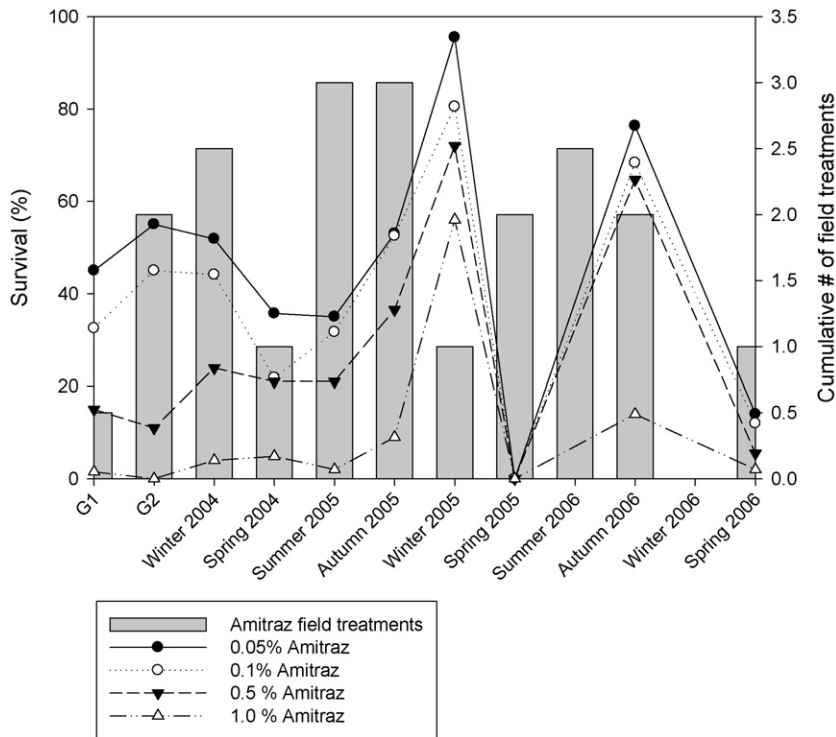


Fig. 2. Percent survival of tick larvae after laboratory exposure to amitraz using the LPT and the number of field applications of amitraz prior to collection in that season. G1 and G2 refer to the 1st and 2nd generation, respectively, after the initial infestations.

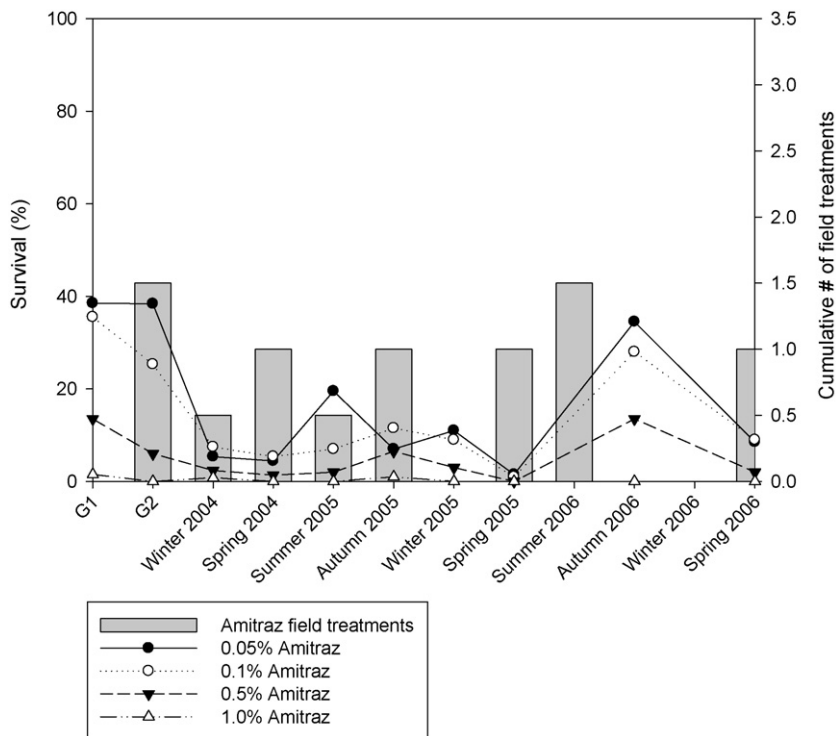


Fig. 3. Percent survival of tick larvae after laboratory exposure to amitraz using the LPT and the number of field applications of amitraz rotated with spinosad prior to collection in that season.

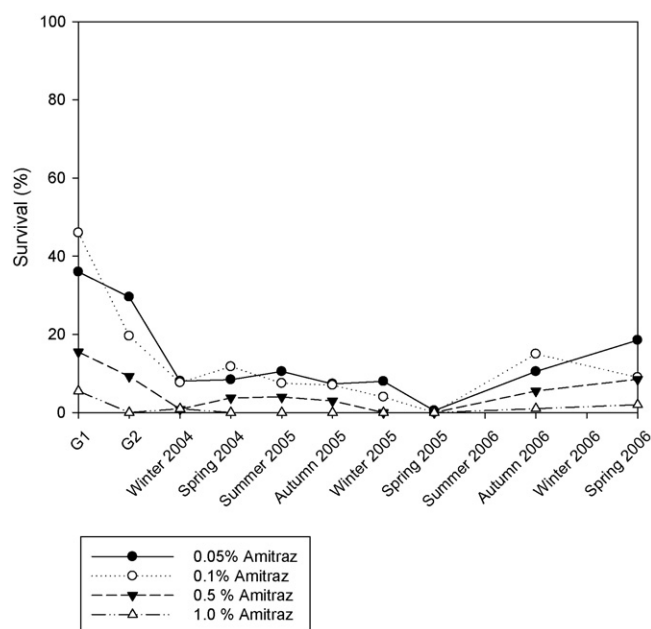


Fig. 4. Percent survival of tick larvae after laboratory exposure to amitraz using the LPT on amitraz-resistant ticks exposed to spinosad under field conditions for 4 years prior to collection.

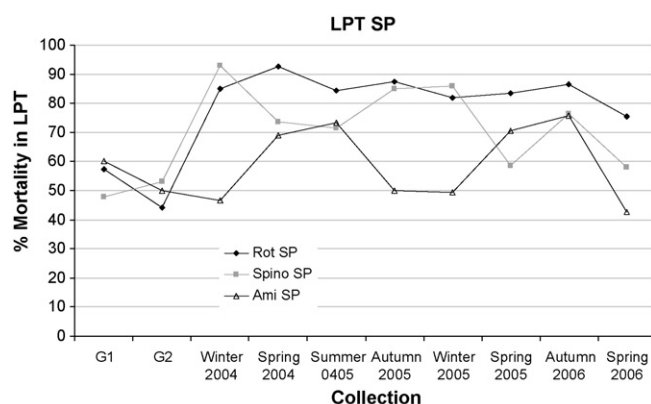


Fig. 5. Mean percentage mortalities of larval progeny of ticks collected from each of the treatment groups on 10 occasions during the trial, when exposed to the discriminating concentration of cypermethrin (0.3%) in the LPT.

similar levels of acceptable efficacy at all concentrations, with low survival ($P > 0.1$). In *Autumn 2006* the percentage survival was again higher in the amitraz-treated groups, significantly so at the 0.5% amitraz concentration ($P < 0.05$), and tending that way in the other concentrations ($P < 0.1$). Finally, in *Spring 2006*, efficacy in the amitraz-treated groups had again returned to similar

levels as the spinosad and the rotation groups ($P > 0.5$). These results indicate that resistance to amitraz increased during spring, summer and autumn, but declined during winter.

Resistance to synthetic pyrethroids did not follow the same marked pattern, but tended to be associated with resistance to amitraz. In the initial phases of the study,

Table 2

Descriptive statistics for the number of acaricide applications required in each treatment group.

Treatment	N	Mean	Median	SD	SE mean
Spinosad	2	25.50	25.50	4.95	3.50
Amitraz	2	20.000	20.000	0.000	0.000
Rotation	2	19.00	19.00	1.41	1.00

Table 3

Analysis of variance for the number of acaricide applications required in each treatment.

Source	DF	SS	MS	F	P
Treatment	2	49.00	24.50	2.77	0.208
Error	3	26.50	8.83		
Total	5	75.50			

immediately after infestation, this would be expected given that the amitraz-resistant ticks used in the artificial infestations were resistant to SPs as well.

4. Discussion

In the amitraz treatment group there was a cyclical increase in resistance to amitraz during summer and autumn, with a reduction in survival in spring and early summer. Figs. 2 and 3 indicate that there is a delay of about one season from the high point in treatment frequency to the peak survival in the LPT. This would be expected, given that the LPT measured the response of the larval offspring from the progeny of the generations that had just been treated. The intergenerational period in south-eastern Australia ranges from 2 to 3 months (Sutherst and Comins, 1979), consistent with this lag. The lowest levels of resistance to amitraz (indicating the lowest frequencies of resistant ticks in the populations) were always noted at the spring collection of ticks. It is impossible to separate the effects of winter conditions from the reduced acaricide application frequency during the same period, however it is conceivable that there might be a direct effect of cold conditions on the relative fitness of amitraz-resistant ticks (in the absence of selection with amitraz). Such an effect has been documented in *Myzus persicae* aphids, in which high levels of expression of esterase-based insecticide resistance was correlated with maladaptive behaviour that reduced survival in winter (Foster et al., 1997). Controlled laboratory studies using all stages of amitraz-resistant and susceptible ticks would be required to confirm an interaction between resistance status and fitness. Under standard tick culture conditions ($30 \pm 2^\circ$, 92.5% RH), one previous study found no consistent differences in engorged weight or egg production in amitraz-resistant and susceptible ticks (Li et al., 2005).

The application of spinosad as an alternating treatment with amitraz and the use of spinosad alone reduced the proportion of amitraz-resistant ticks in the field, in a population that had been generated with moderate levels of resistance to amitraz. Because the experimental design ensured that there were fewer treatments with amitraz in the rotation groups, we cannot conclude that the effect of that treatment is due to rotation as such, rather than simply reduced frequency of application of amitraz. Nonetheless, this finding suggests that ticks with resistance to amitraz are at a selective disadvantage compared with ticks that are not resistant to amitraz.

To date, loss of resistance to amitraz has not been documented after removal of selection pressure. The only study known to us that examines the effect of selection with amitraz in the field is that of Rosado-Aguilar et al. (2008), in which a monthly application of amitraz was shown to increase resistance ratios from one or two times to over 10 times within 15 months. What happened after that was not reported.

We found that spinosad, despite having a lower efficacy than amitraz, could be used to provide effective control of ticks in the field. While it is expected that resistance would rapidly develop to an acaricide with low efficacy, rotation with amitraz could have the desirable effect of lengthening the time to the onset of resistance in spinosad. Although

there is a remote possibility that spinosad treatment specifically selects against amitraz-resistant ticks, the use of virtually any acaricide in a rotation with amitraz might prove to be sustainable. Rotation strategies using amitraz should be developed with caution, however, because secondary mutations could negate the measured loss in amitraz resistance shown in this study. It should also be demonstrated that a similar loss of resistance is measurable in the geographical location where the rotation program will be implemented. Areas with little seasonal climatic variation might not drive similar selection pressures against amitraz-resistant ticks.

Our data from this study show no clear pattern of change in frequency of resistance to SPs in the absence of treatment with SPs. The mortality at the discriminating concentration for cypermethrin ranging from 45 to 60% at the commencement of trial was similar to the 40–80% at the end of the study (Fig. 5). Similarly, resistance to SPs in the field in Queensland currently stands at about 50% of farms, although SPs are only used by about 15% of farmers (QDPI&F YVL records). Roulston et al. (1981) found in 1976–1977 that although chlorinated hydrocarbons were banned from use in Australia in 1962, 49% of farms in south-eastern Queensland showed resistance to dieldrin, indicating that resistance to this class of compounds is not lost with removal of selection pressure.

5. Conclusions

Spinosad 250 ppm provided effective control of amitraz-resistant tick populations in the field, using a statistically similar number of treatments as in the amitraz and rotation groups. Although amitraz provided effective control in the field, there were clear and consistent indications of loss of efficacy using the sensitive larval packet test. Treatment with spinosad or with a rotation between spinosad and amitraz every 2 months resulted in the loss of evidence of amitraz resistance on the LPT and a return to full or almost full susceptibility to amitraz. The loss of resistance to amitraz suggests that rotation of amitraz with other acaricides might prolong the useful life of the product.

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